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14. ABSTRACT

Purpose: Determine in a mouse model of high and low fear whether a protein expressed in the lateral amygdala is more abundant in the high fear mice following Pavlovian fear conditioning Design: Behavioral experiments: compare freezing in naïve, tone alone, and Pavlovian fear conditioned mice during training, context test and cue test. Pharmacological inhibition of pMAPK Measure of key protein: quantify pMAPK-expressing neurons in the lateral amygdala in naïve, tone alone, and fear conditioned mice and create density plots of those pMAPK expressing neurons for comparison across groups Methods: Pavlovian fear conditioning, immunohistochemistry, Western blot, pharmacologically inhibit pMAPK via intraperitoneal injection prior to fear conditioning Sample: adult male High and Low Fear Phenotype mice Analysis: Freeze Frame automated scoring system verified by investigator scoring of freezing. Neurolucida software for quantification of pMAPK expressing neurons in the lateral amygdala; Origin software for the creation of density plots. GraphPad Prism for statistical analysis (ANOVA and t-tests) Findings: High fear phenotype mice exhibit greater freezing (fear) compared to low fear mice during training, context test, and cue test. High fear phenotype mice have more pMAPK expressing neurons in a discrete subregion of the lateral amygdala called the dorso-lateral amygdala (LAd) following Pavlovian fear conditioning. No differences exist between controls. Fear conditioned mice (high and low fear) have more pMAPK expressing neurons in the Lad compared to controls. Inhibition of pMAPK prior to fear conditioning reduces high fear freezing to the level of (and below) low fear freezing. Fear conditioned high fear mice have a unique pattern of pMAPK expressing neurons compared to low fear mice. Implications for Military Nursing: Improved understanding of the cellular and molecular mechanisms that underlie high fear, (a component of PTSD) will aid in the treatment of this neurobiological illness and improve quality of life for nurses and patients.

15. SUBJECT TERMS

fear memory, PTSD, high fear, neurobiological illness, fit and ready force

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Abstract
Purpose: Determine in a mouse model of high and low fear whether a protein expressed in the lateral amygdala is more abundant in the high fear mice following Pavlovian fear conditioning

Design: Behavioral experiments: compare freezing in naïve, tone alone, and Pavlovian fear conditioned mice during training, context test and cue test. Pharmacological inhibition of pMAPK
Measure of key protein: quantify pMAPK-expressing neurons in the lateral amygdala in naïve, tone alone, and fear conditioned mice and create density plots of those pMAPK expressing neurons for comparison across groups

Methods: Pavlovian fear conditioning, immunohistochemistry, Western blot, pharmacologically inhibit pMAPK via intraperitoneal injection prior to fear conditioning

Sample: adult male High and Low Fear Phenotype mice

Analysis: Freeze Frame™ automated scoring system verified by investigator scoring of freezing. Neurolucida software for quantification of pMAPK expressing neurons in the lateral amygdala; Origin software for the creation of density plots. GraphPad Prism™ for statistical analysis (ANOVA and t-tests)

Findings: High fear phenotype mice exhibit greater freezing (fear) compared to low fear mice during training, context test, and cue test. High fear phenotype mice have more pMAPK expressing neurons in a discrete subregion of the lateral amygdala called the dorso-lateral amygdala (LAd) following Pavlovian fear conditioning. No differences exist between controls. Fear conditioned mice (high and low fear) have more pMAPK expressing neurons in the LAd compared to controls. Inhibition of pMAPK prior to fear conditioning reduces high fear freezing to the level of (and below) low fear freezing. Fear conditioned high fear mice have a unique pattern of pMAPK expressing neurons compared to low fear mice.

Implications for Military Nursing: A major component of post-traumatic stress disorder (PTSD) is fear. Recent literature suggests that individuals with PTSD exhibit high and prolonged fear responses. Little is known about the cellular and molecular mechanisms that drive high fear response. Understanding the neurobiological underpinnings of high and prolonged fear memory is important for improved treatment of PTSD and other fear-related disorders.   Findings from these experiments provide evidence that pMAPK, a protein that is known to be required in the LA for the formation of long-term fear memories is more abundant in high fear mice following Pavlovian fear conditioning.   Further, this increased expression of pMAPK is found only in a discrete subregion of the LA, the LAd and when it is pharmacologicaly inhibited in high fear mice, fear memory strength is reduced to the level of low fear mice. Military nurses suffer from PTSD and we also care for patients with PTSD. Improved understanding of the cellular and molecular mechanisms that underlie high fear, (a component of PTSD) will aid in the treatment of this neurobiological illness and improve quality of life for nurses and patients.

TSNRP Research Priorities that Study or Project Addresses

Primary Priority

Force Health Protection:	☒ Fit and ready force ☐ Deploy with and care for the warrior ☐ Care for all entrusted to our care
Nursing Competencies and Practice:	☐ Patient outcomes ☐ Quality and safety ☐ Translate research into practice/evidence-based practice ☐ Clinical excellence ☐ Knowledge management ☐ Education and training
Leadership, Ethics, and Mentoring:	☐ Health policy ☐ Recruitment and retention ☐ Preparing tomorrow's leaders ☐ Care of the caregiver
Other:	☐

Secondary Priority

Force Health Protection:	☐ Fit and ready force ☐ Deploy with and care for the warrior ☒ Care for all entrusted to our care
Nursing Competencies and Practice:	☐ Patient outcomes ☐ Quality and safety ☐ Translate research into practice/evidence-based practice ☐ Clinical excellence ☐ Knowledge management ☐ Education and training
Leadership, Ethics, and Mentoring:	☐ Health policy ☐ Recruitment and retention ☐ Preparing tomorrow's leaders ☐ Care of the caregiver
Other:	☐

Progress Towards Achievement of Specific Aims of the Study or Project

Findings related to each specific aim, research or study questions, and/or hypothesis:

Specific Aim 1: "Determine with quantitative measures whether the acquisition of high or low fear load is associated with increased numbers of neurons or different concentrations of the plasticity associated proteins (pMAPK) in the lateral amygdala". See attached manuscript. We found that High line mice have more pMAPK-expressing neurons in the dorsolateral amygdala (LAd), a discrete subdivision of the LA not at baseline but following fear conditioning. This finding suggests that precise network differences exist between the two fear phenotypes

Specific Aim 2: "Determine whether there is a unique pattern of pMAPK-expressing neurons in the lateral amygdala in high vs. low fear phenotype mice." Our data suggest that High line mice have a unique pattern of pMAPK-expressing neurons within the LAd compared to Low line mice and that this may also underlie the high fear memory behavior. The specific 'map' of neurons expressing pMAPK may reveal key underpinnings of what leads to divergent fear memory and consequently, fear response

Specific Aim 3: "Pharmacologically inhibit pMAPK signaling in High fear phenotype mice prior to fear conditioning and evaluate long-term fear memory". See attached manuscript. when we administered a MEK inhibitor that interrupts the MAPK signaling cascade and blocks the phosphorylation of MAPK, High line mice exhibited fear memory strength similar to that of Low line mice. In these experiments we show that one mechanism of high Pavlovian fear memory is increased activity-dependent plasticity in a key locus of fear memory acquisition and consolidation. This initial study of phenotype and fear memory identified potential differences in two types of memory tests (context and cue). Results suggest that contextual fear memory may be more sensitive to pharmacologic inhibition compared to cued fear memory. SL327, a selective MEK inhibitor, reduced freezing in the High line mice to that of the Low line mice. Interestingly, a differential reduction was seen between contextual and cued fear memory as a result of administration of SL327. An unanswered question is whether the hippocampus is more susceptible to the effects of the systemically-administered drug. We quantified pMAPK-expressing neurons in the LAd one hour following cue fear memory test. Results show a significant reduction in the quantity of pMAPK-expressing neurons in the LAd in the SL327 group compared to the vehicle group. Quantification of pMAPK neurons in the hippocampus is suggested for future studies. More studies in animal models of high and low fear are necessary to further test and confirm these findings.

Relationship of current findings to previous findings:
These findings support previous findings that an increased plasticity in the lateral amygdala of mice is associated with increased strength of fear memory. However, because these data were obtained in a unique population of mice, that is, mice that exhibit very high and ver y low fear, all findings are novel and help to inform the field. Previously, pMAPK expression in the lateral amygdala had not been investigated in mice that exhibit distinct fear phenotypes. We found that mice that underwent Pavlovian fear conditioning had more pMAPK-expressing neurons in the dorso-lateral amygdala and that fear conditioned High fear phenotype mice have more pMAPK expressing neurons than fear conditioned low fear phenotype mice. Thus, this finding is informative and novel.

Effect of problems or obstacles on the results: The only obstacle we encountered was in regards to breeding of our line of mice resulting in a low number of mice for the 3rd specific aim (reducing power). However, the mice we obtained were used and data were obtained. While results were dramatic despite the low number of subjects, repeated projects will be necessary and should include at least 10-12 mice per group.

Limitations:
We had fewer mice available for specific aim 3 than we had planned. However, we have submitted a manuscript reporting these data for publication in a peer reviewed journal. Our findings were dramatic and we found

significance despite the low number of subjects for that particular aim. We did not encounter any other significant limitations.

Conclusion:

Using a mouse model selectively bred to exhibit high and low fear, we used Pavlovian fear conditioning to examine pMAPK expression in the LA in these divergent lines of mice. We hypothesized that high fear mice would have greater pMAPK expression in the LA following fear conditioning as compared to low fear mice. Further, we hypothesized that pharmacologic inhibition of pMAPK in high fear mice would reduce fear memory strength to that of low fear mice. To examine these hypotheses, we quantified pMAPK-expressing neurons in the LA at baseline and at one-hour following fear conditioning. Results indicate that after fear conditioning, high fear mice have more pMAPK-expressing neurons in the dorso-lateral amygdala (LAd) a discrete subregion of the LA. We then used a selective inhibitor of the phosphorylation of MAPK prior to fear conditioning and examined its effects on fear memory strength and the quantity of pMAPK-expressing neurons in the LAd. The results indicate that inhibition of pMAPK reduces contextual and cued fear memory in high fear mice, and reduces contextual but not cued fear memory in low fear mice. Additionally, we found a dramatic decrease in pMAPK expressing neurons in the LAd of high fear mice in which MAPK phosphorylation was pharmacologically inhibited. This suggests that the reduced fear memory is due in part to decreased pMAPK in the LAd. These findings suggest that increased plasticity in the LAd is a component of higher conditioned fear responses and begins to explain, at the cellular level, how different fear responders may encode fear memories differently. Ultimately, this understanding may help to identify novel ways for both identifying and treating individuals who have developed fear-related disorders such as PTSD.

Significance of Study or Project Results to Military Nursing

These data provide important insights into the micro network mechanisms in the barin that may lead to different levels of fear response in individuals. Understanding the circuit level cellular and molecular mechanisms that underlie individual variability in far learning is critical for the development of effective treatment of fear-related illnesses such as PTSD. These data suggest that inhibiting a specific protein in a precise brain region in mice may reduce high fear memory to the level of low fear memory. This information may begin to provide foundations for the understanding and eventual treatment of pathological fear. Using a mouse model of very high and very low fear memory to understand cellular mechanisms in specific brain regions involved in forming fear memories may help identify novel ways to predict individuals at risk for fear related illness. This can potentially lead to targeted treatments for fear-related disorders such as PTSD. While MEK inhibitors have been used in human patients for various other medical conditions such as melanoma, this has not been used in individuals experiencing fear-related trauma. The medical and military relevance of our findings lies in the finding that mice selectively bred to exhibit very high associative fear learning (i.e. learning to associate a sensory stimulus with something fear-inducing) exhibit fear memory at or below the level of low fear mice following MEK inhibition. These findings suggest that MEK inhibitors may be an option in individuals who are at risk for fear-related pathology such as PTSD. Identifying potential avenues of treating PTSD in our military population is critically important and research must continue. Investigating long-term effects of MEK inhibition on general health as well as on fear memory strength should be a follow-on study. Likewise, upstream markers of fear learning in the high and low fear mice should be examined for differences between phenotypes. Because this research was conducted in non-human animals, there is no direct significance (of the results) to military nursing clinical practice or policy. As this research begins to be applied to human fear learning and memory, military nursing relevance will relate to positively impacting quality of life.

Changes in Clinical Practice, Leadership, Management, Education, Policy, and/or Military Doctrine that Resulted from Study or Project

None. This research was fundamental bench science research on a rodent model of high and low fear. While our findings are informative and in line with previous findings, we must conduct more research on systemic and targeted pMAPK inhibition before we can consider this as an option for treating human fear pathology. As such, no changes to clinical practice, policy, or military doctrine have occurred as a result of this research.

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Summary of Dissemination

Type of Dissemination	Citation	Date and Source of Approval for Public Release
Publications	McGuire, J. Coyner, J. Johnson , L. R. (2012). Rodent Models of Conditioned Fear: Behavioral Measures of Fear and Memory. In TRP Channel Targets for Drugs and Toxins. Eds. Arpad Szallasi and Tama Biro eds. Humana Press New York, NY.	
Publications in Press	Coyner J, McGuire, J., Parker, C., Ursano, R., Palmer, A., Johnson, LR. 2013. Mice selectively bred for High and Low fear behavior show differences in the number of pMAPK (p44/42 ERK) expressing neurons in lateral amygdala following Pavlovian fear conditioning. *Neurobiology of learning and memory* Coyner J, McGuire JL, Parker CC, Ursano RJ, Palmer AA, Johnson LR (submitted manuscript 2013 *Neuroscience Letters*), *The MEK inhibitor SL327 differentially inhibits contextual and cued fear memory strength in mice selectively bred for high and low fear.*	USU PAO: March 2013 USU PAO: June 2013
Published Abstracts		
Podium		

| Presentations | Coyner, Jennifer, LTC US Army, PhD Candidate, Program in Neuroscience, Uniformed Services University: "Behavioral and Cellular Expression of Fear Memory in High and Low Fear Phenotype Mice" 3rd year Neuroscience Seminar talk, USU 2012

Coyner, Jennifer, LTC, US Army, PhD Candidate, Program in Neuroscience, Uniformed Services University: Public Dissertation Defense, 17July 2013: Differential expression of phosphorylated mitogen-activated protein kinase (pMAPK) in the lateral amygdala of mice selectively bred for high and low fear. Research funded by TSNRP and USU Dept. of Psychiatry and the Center for the Study of Traumatic Stress | |
| --- | --- | --- |
| Poster | | |

Presentations	Coyner, JL; McGuire, JL; Johnson, LR; "A High and Low Fear Mouse Model". USU Neuroscience Open House, 2011, Bethesda, MD.	USU PAO: October 2012
	Coyner, JL, McGuire, JL, Bergstrom, HC, Palmer, AA, Johnson, LR; "High and low fear phenotype mice show differences in phosphorylated (p44/42 ERK) MAPK-expressing neurons in the lateral amygdala" USU Research Days, May, 2012	
	Coyner, JL, McGuire, JL, Ursano, R, Palmer, A, Johnson, LR; "High and low fear phenotype mice show differential expression of phosphorylated (p44/42 ERK1/2) MAPK in the dorso-lateral amygdala following Pavlovian fear conditioning"; Society for Neuroscience Annual Meeting, OCT 2012, New Orleans, LA	
	Coyner, JL, McGuire, JL, Ursano, R, Palmer, A, Johnson, LR; "High and low fear phenotype mice show differential expression of phosphorylated (p44/42 ERK1/2) MAPK in the dorso-lateral amygdala following Pavlovian fear conditioning"; USU Research Days, 2013, Bethesda MD; (Finalist)	

Reportable Outcomes

Reportable Outcome	Detailed Description
Applied for Patent	none
Issued a Patent	none
Developed a cell line	none
Developed a tissue or serum repository	none
Developed a data registry	none

Recruitment and Retention Aspect	Number
Animals Projected in Grant Application	180
Animals Purchased	0
Model Development Animals	0
Research Animals	
Animals With Complete Data	154
Animals with Incomplete Data	0

Final Budget Report

DETAILED BUDGET FOR INITIAL BUDGET PERIOD				FROM 8/1/2011		THROUGH 7/31/2012		

NAME	ROLE ON PROJECT	Cal. Mnths	Acad. Mnths	Summer Mnths	INST.BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Jennifer Coyner	PD/PI	11.4						0
Luke Johnson	Assoc.-I	1.2						0
Cara Olsen	Statistician	0.12						0
SUBTOTALS				⟶				0

CONSULTANT COSTS		
EQUIPMENT (Itemize)		
SUPPLIES (Itemize by category) Animal (mouse) costs ($10,458), Lab Supplies and Reagents ($6,390), Other Miscillaneous Supplies ($1,851)		18,699
TRAVEL		
INPATIENT CARE COSTS		
OUTPATIENT CARE COSTS		
ALTERATIONS AND RENOVATIONS (Itemize by category)		
OTHER EXPENSES (Itemize by category) Analysis Software Service Contract from MBF Bioscience ($4,390)		4,390
CONSORTIUM/CONTRACTUAL COSTS	DIRECT COSTS	
SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD (Item 7a, Face Page)		$ 23,089
CONSORTIUM/CONTRACTUAL COSTS	FACILITIES AND ADMINISTRATIVE COSTS	11,564
TOTAL PROJECT COSTS FOR INITIAL BUDGET PERIOD		$ 34,653

BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD

BUDGET CATEGORY TOTALS	INITIAL BUDGET PERIOD *(from Form Page 4)*	2nd ADDITIONAL YEAR OF SUPPORT REQUESTED	3rd ADDITIONAL YEAR OF SUPPORT REQUESTED	4th ADDITIONAL YEAR OF SUPPORT REQUESTED	5th ADDITIONAL YEAR OF SUPPORT REQUESTED
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*		3,490			
CONSULTANT COSTS					
EQUIPMENT					
SUPPLIES	18,699	11,810			
TRAVEL		1,678			
INPATIENT CARE COSTS					
OUTPATIENT CARE COSTS					
ALTERATIONS AND RENOVATIONS					
OTHER EXPENSES	4,390	32			
DIRECT CONSORTIUM/ CONTRACTUAL COSTS					
SUBTOTAL DIRECT COSTS *(Sum = Item 8a, Face Page)*	23,089	17,010			
F&A COSTS	11,564	8,168			
TOTAL PROJECT COSTS	34,653	25,178			

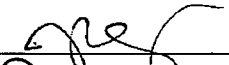| TOTAL PROJECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD	$ 59,831

Additional funds ($14,507) were obtained for the purpose of purchasing an updated high-quality laptop computer for data management, analysis and dissertation writing, for the purpose of funding one additional specific aim that added strength to the overall research methods, and to allow for compensation of a laboratory technician (who is already employed in the lab) for her assistance with daily demands of breeding and weaning mice for the final specific aim.

I requested two rebudgets, one from the "domestic travel" category and one from the "other direct cost" category. For the rebudget from domestic travel, initially budgeted costs were estimations, and my trip actually cost $123 less than expected. For the rebudget from "other direct cost," actual Neurolucida and Stereoinvestigator software costs were charged as "supplies" (i.e. computer software) after being budgeted as "other direct cost" (software maintenance) and therefore the costs in this category were less than expected. The leftover funds were rebudgeted to the supplies category. Finally, HJF indirect costs decreased in FY

2012 and increased in FY 2013, and because of this, adjustments were made to the indirect cost category.

Unspent funds included $4 in the "personnel" category, $1,830 in the "supplies" category, and $122 in the "other direct cost" category.   The HJF accounting systems for payroll rounds the employees' time to the nearest 15 min. resulting in some remaining funds. For "supplies" and "other direct costs," costs were estimated and the actual costs were not exactly the same.

Signatures

PI Signature		Date	01 Nov 2013
Authorized Representative		Date	Nov. 15, 2013

Robert Ursano   for Luke Johnson

Mice selectively bred for High and Low fear behavior show differences in the number of pMAPK (p44/42 ERK) expressing neurons in lateral amygdala following Pavlovian fear conditioning

ABSTRACT

Individual variability in the acquisition, consolidation and extinction of conditioned fear potentially contributes to the development of fear pathology including posttraumatic stress disorder (PTSD). Pavlovian fear conditioning is a key tool for the study of fundamental aspects of fear learning. Here, we used a selected mouse line of high and low Pavlovian conditioned fear created from an advanced intercrossed line (AIL) in order to begin to identify the cellular basis of phenotypic divergence in Pavlovian fear conditioning. We investigated whether phosphorylated MAPK (p44/42 ERK/MAPK), a protein kinase required in the amygdala for the acquisition and

 consolidation of Pavlovian fear memory, is differentially expressed following Pavlovian fear learning in the High and Low fear lines. We found that following Pavlovian auditory fear conditioning, High and Low line mice differ in the number of pMAPK-expressing neurons in the dorsal sub nucleus of the lateral amygdala (LAd). In contrast, this difference was not detected in the ventral medial (LAvm) or ventral lateral (LAvl) amygdala sub nuclei or in control animals.

We propose that this apparent increase in plasticity at a known locus of fear memory acquisition and consolidation relates to intrinsic differences between the two fear phenotypes. These data provide important insights into the micro network mechanisms encoding phenotypic differences in fear. Understanding the circuit level cellular and molecular mechanisms that underlie individual variability in fear learning is critical for the development of effective treatment of fear-related illnesses such as PTSD.

INTRODUCTION

Individual variability in the acquisition, consolidation and extinction of conditioned fear is linked to the pathophysiology of fear disorders including post traumatic stress disorder (PTSD) (26; 58; 89; 91). While the majority of people that experience traumatic events during their lifetime do not develop PTSD, a small percentage of individuals do (64). Pavlovian fear conditioning represents a key model for the study of processes related to PTSD (56; 58; 89). Fear-related pathology may develop in individuals with extreme phenotypes who are highly reactive to traumatic events and slow to recover during extinction of those events (89). On the contrary, resilience, or the ability to adapt to adversity, is suggested to be characterized by low reactivity and fast recovery during extinction (26; 137). As such, PTSD is associated not only with the severity of the trauma (i.e. increased trauma severity increases the likelihood of developing PTSD) but also with the severity of the *reaction* to trauma by an individual (24; 40) (i.e. those with more severe early symptom responses are more likely to develop PTSD). In support of this hypothesis, recent clinical data by Norrholm and colleagues (2011) suggest that persons with PTSD develop a higher 'fear load' in response to the acquisition and consolidation of a new conditioned fear memory (89).

The amygdala is directly implicated in PTSD. Evidence from clinical studies comparing individuals with PTSD to healthy controls shows that those with PTSD have increased amygdala activity to both negative stimuli and to trauma specific stimuli (104). The amygdala is a key brain structure in emotional processing and the various subnuclei that comprise the amygdala play a critical role in the acquisition, consolidation, and behavioral response to associative fear (99; 108). The lateral amygdala (LA) subdivides into three distinct regions called the dorsolateral (LAd), ventral medial (LAvm) and ventral lateral (LAvl). While there are less data relating to the individual roles that these LA subregions play in fear processing, the LAd is proposed to be the

primary locus of sensory and somatosensory synaptic convergence (111). The LAd projects to LAvl and LAvm but LAvl and LAvm do not appear to signal to each other or back to the LAd (99). Thus the LAd is located at the apex of an anatomical and functional network of LA subnuclei. Work in recent decades provide important insight into the cellular and molecular processes underlying the formation of enduring fear memories and Pavlovian fear conditioning has been an invaluable tool in such discoveries (43; 74; 106). This form of classical conditioning pairs a previously neutral cue such as a tone (conditioned stimulus or CS) with an aversive stimulus such as a foot shock (unconditioned stimulus or US). When presented so that one is temporally associated with the other (CS+US), the association is learned and future presentation of the CS alone will elicit a conditioned response (CR) identical to presentation of the US alone.

The LA is the key site for the convergence of sensory stimuli transmitting the CS and US (76; 113). Moreover, the LA is a site for the cellular changes underlying the acquisition and consolidation of Pavlovian fear (74; 79; 93). Memory consolidation and maintenance is the result of plastic changes involving excitatory synaptic transmission and intracellular signaling leading to new protein synthesis (8; 32; 69; 108). Phosphorylated mitogen-activated protein kinase (p44/42 ERK/pMAPK) is required in the LA for the long-term (but not short-term) storage of an associative fear memory through stabilization of long-term potentiation (LTP) from early to the late phase LTP (108; 118). The MAPK cascade involves a series of kinases that lead to downstream activation of transcription factors (such as CREB) and subsequent new protein synthesis (35; 122). This signaling pathway has diverse functions including cell proliferation and differentiation (122), however in the amygdala this pathway is necessary for the long-term consolidation of an associative fear memory (35; 108). In the LA, pMAPK expression is induced in a select population of neurons in response to Pavlovian fear learning (12; 13; 58; 119).

Presentations of the CS or US alone or in a temporally non-paired manner do not result in a Pavlovian fear conditioned memory or in a significant increase in pMAPK neurons (12; 13; 58; 119).

What makes individuals differently susceptible to Pavlovian fear conditioning is an important question for helping to understand individual susceptibility to some anxiety disorders including PTSD. In order to begin to answer this question, it is necessary to understand the cellular basis of divergent Pavlovian fear phenotypes. We began with an F_8 generation C57BL/6J x DBA/2J (B6D2) AIL and selected over three generations to establish divergent mouse lines with a genetic disposition to high and low fear learning after Pavlovian conditioning (94). Mice were selected for high or low contextual and cued Pavlovian fear conditioning (58; 94; 101). Offspring of mice selected for high and low fear (Highs and Lows respectively) were used for parallel behavioral and cellular comparison. To ascertain whether differences in the number of neurons expressing pMAPK correspond to the divergent associative fear learning, we quantified pMAPK neuron numbers in the LA following the induction of Pavlovian fear conditioning in Highs and Lows. This initial examination of whether differential plasticity as measured by pMAPK expressing neurons exists in the LA of divergent lines at baseline and following Pavlovian fear conditioning should be followed by examination of other brain regions within the fear processing circuitry in order to fully elucidate the mechanisms of divergent fear memory.

MATERIALS AND METHODS

Animals

High and Low Pavlovian fear mouse lines

Short-term selection for contextual fear was used to create outbred mouse lines with robust differences in fear learning (101). Mice were phenotyped and selected beginning with the

F_8 generation C57BL/6J x DBA/2J AIL (B6D2 F_8) obtained from the University of Chicago

(94). The mice that exhibited the highest and lowest contextual freezing one day following

Pavlovian fear conditioning were selected to create new breeding pairs in the High and Low

lines, respectively (10-30% each of the High and Low populations to create 12 breeding pairs in

each line). Freezing to the CS was used as an additional measure for selection when necessary.

Siblings and first cousins were never paired for breeding. Offspring were fear conditioned at 8-

10 weeks of age, selected for high and low contextual fear and High and Low line breeding pairs

were again formed. This process continued for 3 selection generations (S1-3). Behaviorally

naïve, adult male mice from the 4th selected generation were used for experiments described

here. Mice were randomly assigned to one of two parallel experimental cohorts: behavior

(BEH), or immunohistochemistry (IHC) and were then further randomly assigned to one of three

groups: Naïve (to US and CS), Tone (CS alone), or Paired (Conditioned, US and CS).

Husbandry

Mice were housed 2-5 per cage segregated by sex and line (High or Low) in standard

shoebox cages in a climate controlled vivarium on a standard 12hr light/dark cycle with ad

libitum food and water. Experiments were conducted during the light cycle. All experiments

adhered to IACUC approved protocols and procedures were conducted in accordance with the

National Institutes of Health *Guide for the Care and Use of Experimental Animals.*

Pavlovian fear conditioning

Forty-eight adult male mice aged 2-8 months were used for all behavior experiments.

Conditioning chambers (Coulbourn Instruments, Whitehall, PA) measured 7"Wx7"Dx12"H and

were inside sound attenuation chambers. Two distinct environments were created with these

chambers in order to represent box "A" and box "B". Alterations to the environments involved

changes in flooring (grid shock floor for training and context test; wire mesh non-shock floor for

cue test), chamber dimensions and appearance, lighting, and scent (70% isopropyl alcohol

cleaning solution versus commercial cleanser). Mice were transported to a holding area free of

high human traffic and noise and they remained in this area for 30 minutes prior to being placed

into the conditioning chambers. Behavior experiments consisted of 3 days of habituation to "A",

training day in "A", context test day in "A", and cue test day in "B" (6 consecutive days, see

figure 1).

Pavlovian fear conditioning consisted of three paired tone/shock presentations

(CS/tone+US/foot shock x3) over 10 minutes with approximately 1-2 minutes between CS+US

pairings (the inter-trial interval or ITI). Tones were 75dB, 5000Hz and lasted 30 seconds.

Shocks were 0.6mA for the final 1 second of the tone. Animals were allowed 3 minutes in the

chamber prior to any stimuli and 2 minutes elapsed following the final tone/shock pairing prior

to removal from the chamber. Mice assigned to the fear conditioning group receiving both CS

and US are referred to as "Paired" (n= 12 Highs and 12 Lows). Mice assigned to the "Tone"

groups (CS only) received training identical to the paired groups, however no shocks were

administered (n= 6 Highs and 6 Lows). "Naïve" animals were placed in the conditioning

chambers for equivalent periods of time with no CS or US (n= 6 Highs and 6 Lows). One day

following fear conditioning, animals were returned to the training context "A" for 10 minutes

and freezing was scored. The next day (two days following training), in order to isolate

associative cued fear, mice were placed into a novel environment "B" and three tones identical to

the training CS in quality and duration were administered over a 10-minute period of time.

Freezing, an active behavior, is the cessation of all movement except for movements associated

with respiration (18). Freezing was measured using FreezeFrame™ (Coulbourn Instruments,

Whitehall, PA) automated scoring and verified by the investigator. Adjustments to threshold were made so as to accurately detect freezing and varied depending on mouse coat color (gray, light brown, or black) and lighting in each chamber.

Scoring of freezing

Freezing was measured at time-matched points across groups as follows: Training Day: during the thirty seconds of tone+/- shock 2 and 3 (or equivalent time points for Naive mice), Context Test Day: during the initial 5 minutes in the chamber, and Cue Test Day: during tone 1, 2, and 3 with baseline novel context freezing subtracted from tone 1 freezing. All data are means per group.

Immunohistochemistry (IHC)

Mice underwent 3 days of habituation to context A followed by training as described in behavior experiments. One hour following training, mice in the IHC cohorts were anesthetized with ketamine/xylazine (100mg/kg + 10mg/kg) via intraperitoneal injection and transcardially perfused (Gravity Perfusion System for mouse, AutoMate Scientific, Berkeley, CA) with 20ml 0.9% normal saline followed by 40ml 4% paraformaldehyde (FD Neurotechnologies, Columbia, MD). Brains were post-fixed overnight and transferred to 1X phosphate buffered saline (PBS) until processing for immunohistochemistry. Brains were sliced on a vibratome at 40µm and marked in a consistent manner to indicate rostro-caudal sequence. Free-floating sections (5 per well) were placed in 1% BSA blocking solution for one hour prior to incubation in rabbit polyclonal primary antibody to phospho-p44/42 MAPK (1:250 dilution, Cell Signaling Technology, Boston, MA) for 24 hours at room temperature. Following 5 washes in PBS,

sections were incubated in secondary antibody (biotinylated goat anti-rabbit IgG, 1:200 dilution, Vector Laboratories) for 30-minutes, washed x4, and then placed in avidin-biotin HRP complex (ABC Elite, Vector Laboratories) for 1-hour. Visualization of pMAPK-expressing neurons was achieved by SG chromagen/hydrogen peroxide (Vector Laboratories). Sections were mounted in sequence on Superfrost ™ slides, allowed to completely dry, and then dehydrated in increasing percentages of alcohol (50%x1, 70%x1, 95%x1, 100%x2) followed by xylene.

Section Alignment

Analysis of pMAPK-expressing neurons was conducted on matched amygdala slices across subjects. Matching sections across subjects was achieved by using consistent, identifiable landmarks and the Franklin and Paxinos "The Mouse Brain in Stereotaxic Coordinates" (46) (see figure 3). The optic tract reliably appears and lengthens in coronal sections placed in rostral to caudal sequence. We matched sections across subjects by using the relationship between the right optic tract and the central nucleus (CeA) of the amygdala (see Figure 3). Sections in this analysis were from bregma -1.58mm, -1.70mm, and -1.82mm. Contours of the lateral amygdala (LA) were traced for each bregma coordinate and applied to each section so as to count from a consistent area that accurately depicts the region of interest as illustrated in the mouse brain atlas.

Statistical Analysis

Data were analyzed using GraphPad Prism (version 5) statistical software. Unless otherwise stated, two-way analysis of variance (ANOVA) with post-hoc t-test was used for each analysis. Data are expressed as mean +/- Standard Error of the Mean (SEM) and significance is defined as $p < 0.05$.

RESULTS

Behavioral results

In the parallel behavioral group both Highs and Lows acquired Pavlovian fear but show differences in the strength of Pavlovian fear acquisition. Measurement of freezing to tone 2 and 3 during the acquisition of Pavlovian fear conditioning ("training", Day 1) revealed that both Highs and Lows acquired Pavlovian fear (figure 2A). A significant interaction between phenotype and experimental group exists $[F (2, 52) = 9.79, p = 0.0002]$. Lows in the Naive and Tone groups did not differ in freezing (mean 1.54 +/- 0.355 % and 3.17 +/- 1.985 %, p = 0.438). However, Lows in the Paired group exhibited significantly more freezing compared to controls (mean 13.18 +/- 2.39%, p = 0.0019). Highs demonstrated a similar response to training across experimental groups. Naive and Tone Highs did not differ in freezing (mean 3.11 +/- 1.265% and 9.04 +/- 3.868%, p = 0.175). However, Paired Highs exhibited significantly more freezing (mean 47.97 +/- 4.10%, p < 0.0001). Importantly, comparison of control groups revealed no difference between Highs and Lows (Naive Highs vs. Naive Lows p=0.258; Tone Highs vs. Tone Lows p = 0.206). Freezing in Paired Highs was greater than Paired Lows (p <0.0001). Both phenotypes freeze during the tone once it has been paired with a foot shock. Thus, at training both Paired Highs and Paired Lows acquire Pavlovian fear memory and Paired Highs demonstrate greater freezing during this acquisition.

High fear mice freeze more upon return to training context A 1 day after training (figure 2B). Measurement of freezing during testing of consolidated contextual Pavlovian fear memory ("context test", Day 2) revealed that both Paired Highs and Paired Lows consolidated a contextual fear memory. However, a difference was seen between the Highs and Lows in the strength of the contextual fear memory. A significant interaction between phenotype and experimental group exists $[F (2, 41) = 3.29, p= 0.047]$. Lows in the Naive and Tone groups did not exhibit a difference in freezing (mean 0.818 +/- 0.610%, and 0.376 +/- 0.257%, p= 0.510).

However, Lows in the Paired group exhibited significantly more freezing compared to controls (mean 12.21 +/- 3.35%, p= 0.0106). High Naive and Tone groups did not differ in freezing (mean 0.506 +/- 0.146, and 0.492 +/- 0.244%, p= 0.962). However, Paired Highs exhibited significantly more freezing than Highs in control groups (mean 40.30 +/- 8.41, p= 0.0020). Comparison of control groups revealed no difference between Highs and Lows (Naive Highs vs. Naive Lows: p= 0.661, Tone Highs vs. Tone Lows: p= 0.755). Importantly, data revealed a difference between Paired Highs and Paired Lows (p= 0.0052). Thus, context test data suggest that both Paired Highs and Paired Lows consolidate a Pavlovian contextual fear memory but that Paired Highs appear to consolidate a stronger contextual fear memory.

High fear mice freeze more upon presentation of the fear-associated cue (figure 2C). Isolating fear to the specific cue was assessed by placing mice in a novel context (B) two days following training and re-administering the CS. Measurement of freezing during testing of consolidated cued Pavlovian fear memory ("cue test", Day 3) revealed that both Paired Highs and Paired Lows consolidated a Pavlovian cued fear memory. However, a difference was seen between the Highs and Lows in the strength of the cued fear memory. A significant interaction between phenotype and experimental group exists [$F_{(2, 42)} = 3.65$, p= 0.035]. Lows in the Naive and Tone groups did not exhibit a difference in freezing (mean 13.82 +/- 6.80%, and 5.54 +/- 3.75%, p= 0.311). However, Lows in the Paired group exhibited significantly more freezing compared to controls (mean 29.76 +/- 5.65%, p= 0.0224). High Naive and Tone groups did not differ in freezing (mean 14.44 +/- 5.44, and 7.06 +/- 5.87%, p= 0.237). However, Paired Highs exhibited significantly more freezing than Highs in control groups (mean 60.62 +/- 6.28, p<0.0001). Comparison of control groups revealed no difference between Highs and Lows (Naive Highs vs. Naive Lows: p= 0.944, Tone Highs vs. Tone Lows: p= 0.734). As in training

and context test, cue test data revealed a difference between Paired Highs and Paired Lows (p= 0.0014). Thus, cue test data suggest that while both Paired Highs and Lows consolidated a cued Pavlovian fear memory, Paired Highs consolidate a stronger fear memory to the cue. In a novel context, presentation of the fear-associated cue resulted in twice as much freezing in the Paired Highs compared to Paired Lows.

Immunochemistry results

Paired mice had more pMAPK-expressing neurons in the LAd compared to control mice and among those, Paired Highs had more pMAPK-expressing neurons than Paired Lows (figure 4A). We quantified pMAPK-expressing neurons in the LA in three matched coronal sections (bregma -1.58mm, bregma -1.70mm, and bregma - 1.82mm) according to the Franklin and Paxinos "The Mouse Brain in Stereotaxic Coordinates" (3rd ed., 2008). All subjects across groups and phenotype had pMAPK-expressing neurons in the LA one-hour following training. A difference in pMAPK expressing neuron number was seen in Paired Highs and Paired Lows in the LAd (two-way ANOVA: [F (2, 30) = 4.05, p= 0.0277]. Lows in the Naive and Tone group did not exhibit a difference in pMAPK-expressing neurons (mean 11.53 +/- 2.070% and 18.33 +/- 2.62%, p= 0.0690). However, Lows in the Paired group exhibited significantly more pMAPK neurons compared to controls (mean 27.73 +/- 2.52, p= 0.0010). Naive and Tone Highs did not differ in pMAPK neuron number (mean 16.00 +/- 2.77% and 21.33 +/- 3.94%, p = 0.294). However, Paired Highs exhibited significantly more pMAPK neurons (mean 50.26 +/- 6.85%, p = 0.0003). Comparison of control groups revealed no difference in pMAPK neuron numbers between Highs and Lows (Naive Highs vs. Naive Lows p=0.225; Tone Highs vs. Tone Lows p = 0.5406). Importantly, data revealed a difference in pMAPK neuron number between Paired Highs and Paired Lows (p = 0.0115). Quantification of pMAPK-expressing neurons in the LAvm

did not reveal a difference among experimental group or phenotype [F (2, 30) = 0.84, p = 0.441],

[F (2, 30) = 1.62, p = 0.213] (figure 4B). Quantification of pMAPK-expressing neurons in the

LAvl did not reveal a difference among experimental group or phenotype [F (2, 30) = 1.40, p =

0.260], [F (2, 30) = 0.76, p = 0.389] (figure 4C).

DISCUSSION

We used a B6D2F$_8$ advanced intercross (C57B6/6J and DBA/2J) mouse line selected

over 3 generations for differential expression of contextual and cued Pavlovian fear (101). We

investigated endogenous differences in cellular plasticity in the lateral amygdala between the

phenotypes. We determined that pMAPK, a protein required for the consolidation of Pavlovian

fear memory, is differentially expressed in the LA of mice expressing divergent Pavlovian

conditioned fear. Systematic matching of the rostral to caudal location of amygdala containing

brain sections allowed for quantitative measurement of pMAPK expressing neuron numbers

within the three sub nuclei of the LA (LAd, LAvl, LAvm). No differences in pMAPK neuron

number were found in either the LAvm or the LAvl between the control and paired (Pavlovian

fear) groups or between High and Low fear phenotypes. In contrast, a significant difference in

pMAPK expressing neuron number was found in the LAd both between control and paired

group, and also between the High and Low fear phenotypes. Thus, a difference in pMAPK

expression mediated by different numbers of neurons expressing pMAPK is associated with a

genetically selected differential expression of Pavlovian fear. These data provide important

insights into the micro network mechanisms encoding both normal and potentially pathological

fear.

The advantage of using an AIL over comparisons of inbred mouse strains differing in fear

memory behavior is a that a tighter association of genes and traits can be made from the highly

recombinant AILs (10; 92; 94; 101). Selected animal lines developed from an AIL allow for

increased specificity in isolating genetically determined behavioral and physical phenotypes due

to a breakdown in linkage disequilibrium (58; 92; 94). Traits unassociated with the behavior of

interest remain evenly distributed between the lines while relevant traits will segregate with the

behavior (92; 94). Previous studies in the B6D2 AIL identified several narrow chromosomal

regions and possible genes underlying the behavioral responses (92). The reduction of behavioral

differences between the selected lines to fear and anxiety related behaviors is intended to isolate

cellular mechanisms that drive the phenotypes and make them more likely to be identifiable (58;

92; 94).

AIL alleles originate in the founder strains. An early study by Paylor and colleagues

(1994) (95) suggested that the DBA/2J (D2) strain has a hippocampal deficit when compared

against the C57BL/6J (B6) strain. They found impaired hippocampal dependent contextual fear

conditioning and no difference in amygdala dependent cued fear conditioning. A more recent

study by Balogh and colleagues (9) confirmed that the D2 show less freezing to the same

contextual shock paradigm compared to B6. In addition they showed that B6 show more

contextual fear generalization and poor discrimination of contexts compared to the D2. These

two studies (9; 95) show the fundamental differences in fear behavior between the strains. In our

B6D2 F_8 AIL mice it is possible that aspects of these D2 characteristics are transmitted to the

Low fear mice whereas the B6 characteristic are transmitted to the high fear mice. If this is the

case a key question is which mechanisms within the network are modified by the transmitted

genes (56; 74).

Importantly, the 8 generation AIL has extensive allele fragmentation (94). Thus the

intercrossed mice and the subsequent segregated lines contain alleles transmitted from the

foundation lines but likely contain novel allele combinations. These novel combinations may result in novel phenotypes. Importantly we observe that our High and Low fear lines show different levels of both contextual and cued fear (Figure 2) unlike the B6 and D2 which differ only on contextual fear (9; 95). The foundation strain also shows other behavioral and physiological differences possibly contributing to the novel High and Low fear B6D2 line. Moreover, differences in fear responses between the B6 and D2 founding strains include both freezing and autonomic responding. Steidl and colleagues (128) compared both C57BL/6J (6J) and 6N (6N) with the DBA/2J (2J) and the 2N (2N). They identified that not only do the 2J and 2N show less freezing than the 6J and 6N they also showed reduced heart rate activation compared to the 6J and 6N. These data suggest that the differences in fear are likely centrally mediated by amygdala. Amygdala afferents regulate both freezing and autonomic responses (56; 74). These data are important because they also point to the amygdala as a key site in the selected lines for physiological manifestations of genetic differences transmitted by B6 and D2 founding strains.

Little is known about the cellular and molecular mechanisms that underlie high and low fear response and yet, the underpinnings of these divergent responses must be understood in order to effectively treat fear-related illness (58). Data from these experiments indicate that High fear mice have more pMAPK-expressing neurons in the LAd following Pavlovian fear conditioning (figure 4). First, High fear mice potentially have a more dense fear network involving more neurons within the lateral amygdala fear circuit. If this is the case, the increase in pMAPK expressing neurons observed may be a function of increased total neuron population. A second possibility is that the actual neuron numbers are equivalent between the High and Low fear lines, but that amygdala afferent synaptic strength is increased leading to more pMAPK

expression per neuron. A third possibility is that there are differences in upstream effectors of the

MAPK signaling cascade such as MEK (MAPK kinase) and Raf (MEK kinase) leading to

differences in efficiency or sensitivity of the MAPK cascade between High and Low animals

(35; 122). Finally, in LA neurons the MAPK cascade is induced through neuronal calcium influx

and depolarization (108). Differences between High and Low animals in the number of pMAPK

neurons may therefore reflect functional differences in membrane receptors or ion channels of

LAd neurons between High and Low lines.

Differences in pMAPK expressing neuron numbers between High and Low line mice

were specific to the LAd. The anatomical specificity of these differences further supports the role

of the LAd as the apex in a pathway of plasticity within the amygdala following Pavlovian fear

conditioning. Repa and colleagues (2001) identified two different populations of neurons

exhibiting plasticity within the LAd following Pavlovian fear. First, a population of neurons in

the dorsal tip of the LAd exhibited transiently plastic neurons. In contrast, a second population

was identified in the ventral LAd that exhibited long term plasticity. These differences may have

been driven by differences in afferent connectivity with the thalamus targeting the short-term

plastic neurons while cortical and intra-amygdala projections may target the ventral long term

plastic LAd neurons (105). We previously identified an intra-LAd circuit that supports a role for

feedback-mediated plasticity within the LAd (54; 57). While plastic changes have also been

identified in the LAvm and LAvl (12), studies of LA plasticity and connectivity suggest the LAd

is the key site for initiation and storage of amygdala dependent plasticity. We recently showed in

a rat model that Pavlovian fear conditioning is precisely localized to a population of neurons

within discrete and stable micro regions within the LAd (12; 13). Importantly anatomical studies

suggest that connectivity and communication among the LA subdivisions is unidirectional from

LAd to LAvm and LAvl. Further no signaling is reported between LAvm and LAvl (100).

Projections from within the amygdala to the LA terminate in LAvm and LAvl (100). Projections

from the LA to the amygdala predominately originate in the LAvm (100). These functional and

anatomical studies suggest that the LA sub divisions have a unique role in storage and processing

of Pavlovian fear memories with the LAd at the apex of this circuit.

The finding that differences in pMAPK expressing neuron number are precisely localized

to an LA sub nucleus in High and Low line mice suggests precise micro network differences

between the two fear phenotypes. The approximate percentage of glutamatergic and GABAergic

neurons in the LA in rat is 85% and 15% respectively (110). The total numbers of pMAPK

expressing cells in the LAd occurs in approximately the same ratio (87% of pMAPK+ cells co-

express CAMKIIa) (12). While these data in rat have not yet been confirmed in mice, it seems

likely that, as in rat, the majority of fear learning induced plasticity in the LA is occurring in

excitatory neurons. Following associative fear learning the High selected line mice have more

pMAPK expressing neurons in the dorsolateral amygdala (LAd) compared to the Low selected

lines. This experience-induced plasticity occurs within a key region of interest within the LAd

(12; 13; 54; 56; 57; 105) where fear-associated auditory and somatosensory information

converge within the amygdala (52; 57; 76; 113). Mice in control groups show equivalent

numbers of pMAPK expressing neurons and demonstrate that the increase in the pMAPK cell

population is related to the learned CS-US association (figure 4). While these data alone are

insufficient to establish this as a mechanism for increased fear memory, the data are compelling

and provide important information for future experiments.

The mechanisms driving the differences in pMAPK neuron numbers are an important

target for future research. Upstream regulators of calcium signaling, particularly AMPA

receptors (116), are key targets for future research in these mice and similar models. Benedetto et al 2009 have proposed the use of pMAPK targeted drug therapy for use in anxiety disorders based on identification of the essential nature of ERK/pMAPK in both the consolidation and reconsolidation of fear memory (12; 13; 39; 58; 118). The present data showing that pMAPK expression increases after Pavlovian fear learning, also demonstrates the differential role of pMAPK in the relative strength of Pavlovian fear in a genetically heterogeneous population. The AIL fear phenotype mice are thus a useful model for testing ERK/MAPK-targeted pharmacotherapy for treatment of Pavlovian based aspects of fear pathologies (38; 58). An important follow-up experiment will be to pharmacologically inhibit pMAPK in both lines prior to fear conditioning and examine the behavioral and histochemical effects within and between High and Low line mice. Additionally, investigation of pMAPK-expressing neurons in other brain regions within the fear circuit such as BA, Ce, auditory thalamus, hippocampus, and prefrontal cortex may reveal other important differences between the lines that explain the divergent fear memory behavior.

The diagnosis of PTSD requires a set of criteria be met including experience of threat to life or the witnessing of this in others, prolonged hypervigilance, avoidance of reminders of trauma, and intrusive thoughts about the experience (6). While much is understood about the symptomatology of PTSD, little is known about the neurobiological mechanisms that underlie this devastating illness. It is unclear whether an increased conditioned response to fear stimuli is a consequence of or a predictor of PTSD (96). Recent prospective studies indicate conditioned fear may be a predictor of Post-Traumatic Stress Syndrome (PTSS) in firefighters (91). Some people may have pre-trauma vulnerabilities making them susceptible to posttraumatic stress and potentially more likely to develop PTSD (91). These data for PTSD support a body of evidence

that stronger acquisition of conditioned fear is common to anxiety disorders (77). However, processes at the cellular and molecular level underlying the variable responses that lead to high reactivity and subsequent fear pathology are unknown. Therefore, identification of the neurobiological mechanism underlying differences in the acquisition of conditioned fear will aid the understanding of normal and pathological fear (26; 55; 89; 91).

Recent clinical data suggest that persons with PTSD develop a higher 'fear load' in response to the acquisition and consolidation of a new conditioned fear memory (89). Importantly, PTSD may thus be associated less with the severity of the trauma, and more with the severity of the reaction to trauma by an individual (24; 40). Individuals that exhibit high fear response during acquisition of a fear memory as well as when exposed to contextual and cued reminders of the fear experience may be the 'at risk' population for future development of PTSD. Mouse models of high and low Pavlovian conditioned fear are therefore an important tool in the further discovery of mechanistic differences between individual variability in fear response and more efficacious treatment of fear-related illness.

In summary, these data provide important biological data on the cellular mechanisms of differences in conditioned fear between High and Low fear mice. Non-human animal models offer valuable insight into understanding the neurobiological mechanisms that underlie individual variability in acquisition and consolidation of fear memory (37). Because fear circuitry is conserved across species, the cellular and molecular mechanisms of human fear can be identified using non-human animals such as rodents (74). Selectively bred animal models that isolate distinct behavioral phenotypes such as very high or very low fear are a valuable tool to the study of how mammals learn fear. Further, examining extremes in fear behavior can better suggest mechanisms and lead to more effective treatment of fear-related illness in humans. Data

from the present study may help provide important insights into individual differences in

Pavlovian fear and how differences in pathological fear are established (58).
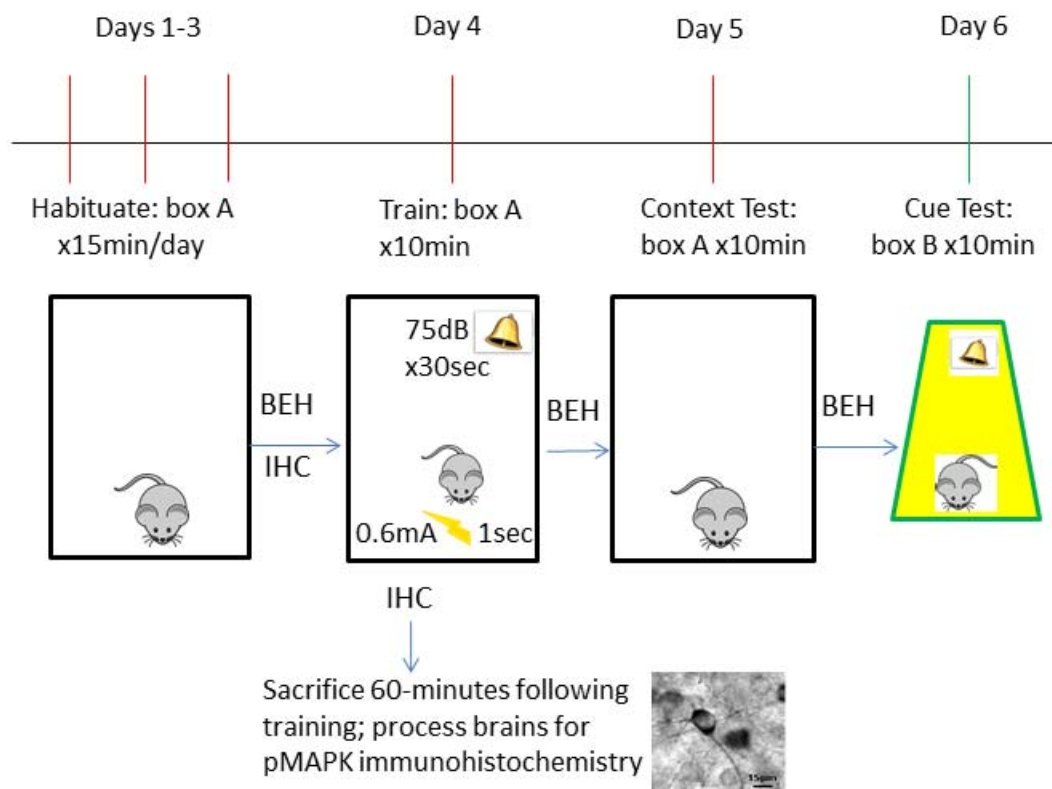

Figure 2.1. Experimental design for comparison of phenotypes in response to Pavlovian fear
     conditioning and analysis of pMAPK expression. Mice habituate to the conditioning
     chamber (A) for 15-minutes a day for three consecutive days immediately preceding
     training. All cohorts (Naïve, Tone alone, and Paired) undergo habituation to box A. On
     day four, mice receive training as indicated by random group assignment. Paired group

animals receive 3 tone/shock pairings, Tone group animals receive identical training to the paired group without shocks. Naïve animals spend the same amount of time in the box with no tones or shocks. Subjects assigned to IHC are sacrificed 60-minutes following training and brains are subsequently processed to detect pMAPK-expressing neurons. One day after training, BEH subjects are returned to the training context (A) for context testing. On the final day of behavior experiments, subjects undergo cue testing during which they are placed in a novel context (B) and administered three tones identical in duration and quality to the training CS.
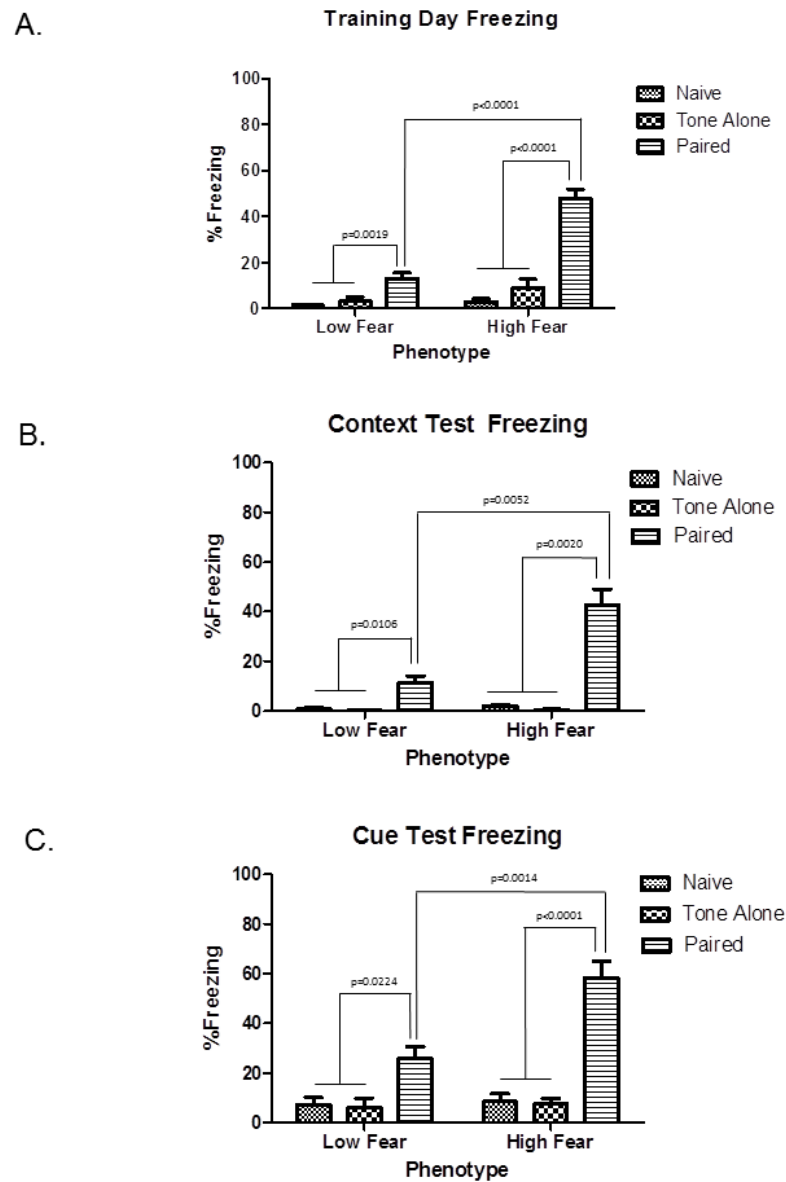
A.

B.

C.

Figure 2.2. High and Low Pavlovian fear mice show differences in fear acquisition, context test, and cue test. (A) Training Day: High fear mice exhibit greater freezing relative to Low fear mice that receive identical training (p<0.0001). Low and High mice that received tone/shock pairings freeze more than control groups. Control groups do not differ. (B) Context Test Day: Both Low fear and High fear mice exhibit fear to the context. Fear conditioned High mice freeze more than fear conditioned Low mice suggesting stronger contextual fear memory consolidation. Tone group High mice exhibited less freezing than High naïve mice but differences are not significant (C) Cue Test Day: High mice exhibit greater freezing to the fear-associated auditory cue relative to Low mice.
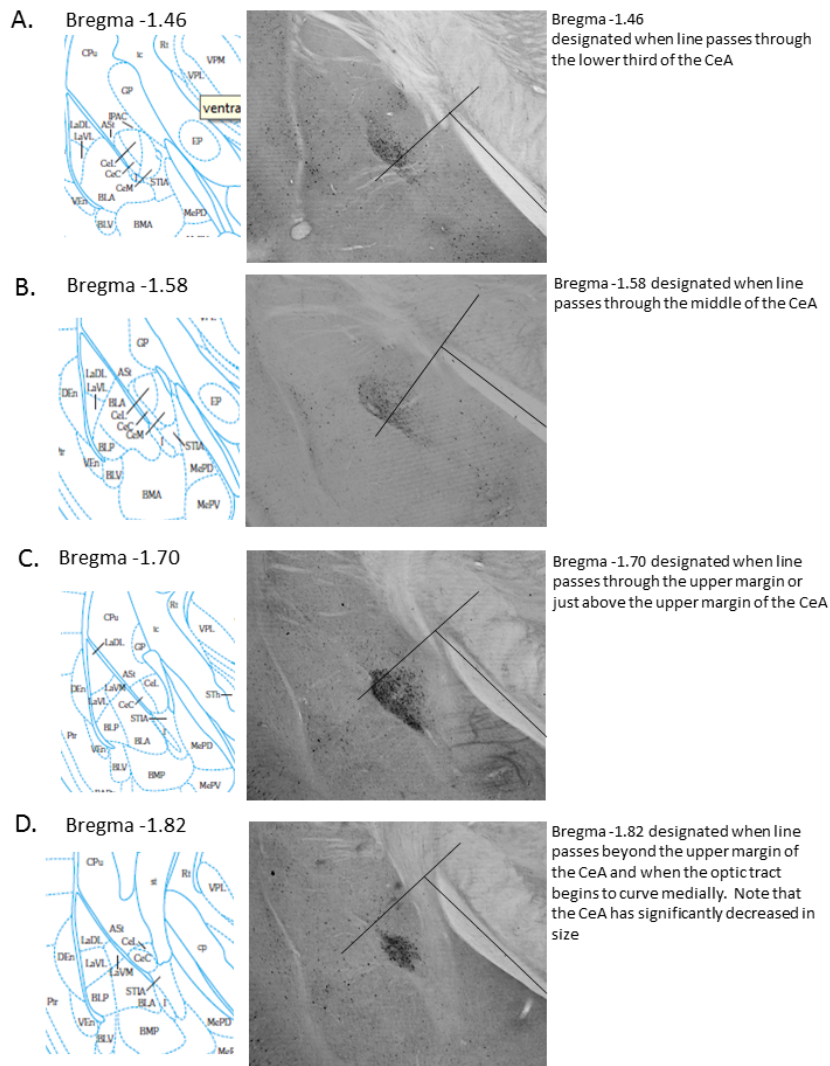
A. Bregma -1.46
Bregma -1.46 designated when line passes through the lower third of the CeA

B. Bregma -1.58
Bregma -1.58 designated when line passes through the middle of the CeA

C. Bregma -1.70
Bregma -1.70 designated when line passes through the upper margin or just above the upper margin of the CeA

D. Bregma -1.82
Bregma -1.82 designated when line passes beyond the upper margin of the CeA and when the optic tract begins to curve medially. Note that the CeA has significantly decreased in size

Figure 2.3. Anatomical demarcation of rostro-caudal location of lateral amygdala brain slices for pMAPK immunohistochemistry. Slices were compared to the Franklin and Paxinos mouse brain atlas (2008 at left, used with permission) for verification of the right central nucleus (CeA) in relation to the right optic tract. (A) Bregma -1.46 slices were designated when the right optic tract terminated at the lower third of the CeA and when the CeA was round in shape (not used for neuron quantification). (B) Bregma -1.58 slices were designated when the CeA appeared as a teardrop shape and the optic tract terminated at approximately the middle of the CeA. (C) Bregma -1.70 slices were designated when the optic tract was level with or extended just beyond the CeA but prior to when the optic tract curved medially. (D) Bregma -1.82 slices were designated when the optic tract extended beyond the superior border of the CeA and when it curved medially while retaining specific margins.
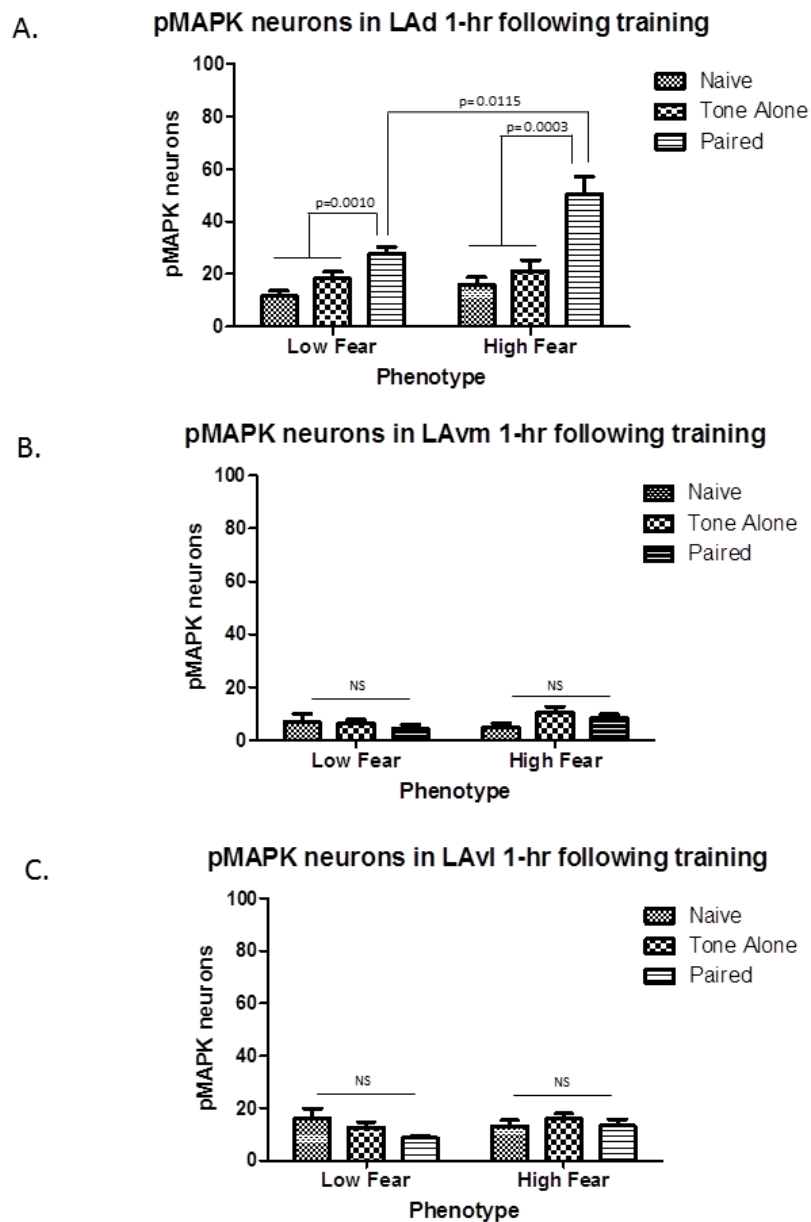
A.

B.

C.

Figure 2.4. Following Pavlovian fear conditioning, High and Low fear mice have different numbers of pMAPK-expressing neurons in the LAd. (A) Total pMAPK-expressing neurons counted in the LAd in Highs and Lows in each experimental group. (B) Total pMAPK-expressing neurons counted in the LAvm in Highs and Lows in each experimental group. (C) Total pMAPK-expressing neurons counted in the LAvl in Highs and Lows in each experimental group. pMAPK-expressing neurons were quantified in the LA at 40x objective.

The MEK Inhibitor SL327 differentially inhibits contextual and cued fear memory strength in mice selectively bred for high and low fear

ABSTRACT

Pavlovian fear conditioning is a widely used amygdala-dependent model of fear memory. However, the neurobiological mechanisms underlying fear memory strength are less well understood. Understanding the neurobiological mechanisms of fear memory strength is important in order to better understand disorders of fear memory including Post traumatic stress disorder (PTSD). PTSD is a serious medical condition affecting both military and civilian populations. While its etiology remains poorly understood PTSD is characterized by high and prolonged levels of fear response. Here, we used a selected mouse line of High and Low Pavlovian conditioned fear to begin to identify aspects of the cellular basis of phenotypic divergence in fear memory strength. We have previously demonstrated differences in the number of phosphorylated MAPK/ ERK (pMAPK) expressing neurons in the dorso-lateral amygdala (LAd) of these High and Low fear mice. Here we used a selective MEK inhibitor (SL327) to pharmacologically inhibit pMAPK/ERK prior to fear conditioning and examined fear memory strength and the quantity of pMAPK-expressing neurons in the LAd. We found contextual fear was abolished in both High Fear and Low Fear mice. In High fear mice, while contextual fear is completely abolished by SL327, cued fear was only reduced to ~50% of its control. In High fear mice we found SL327 reduced the number of neurons expressing pMAPK/ERK in the LAd. These data suggest that contextual fear is more sensitive to disruption by the MEK inhibitor SL327 than cued fear. Collectively these data support the hypothesis that different levels of the expression of pMAPK/ERK may contribute to the behavior of high and low fear individuals. These data suggest that different numbers of amygdala neurons undergoing phosphorylation of

ERK1/2 kinases contribute to different strengths of conditioned fear. These data begin to provide

foundations for the understanding and eventual treatment of pathological fear. This

understanding may help identify novel ways to predict individuals at risk for fear-related illness

and can potentially lead to targeted treatments for fear-related disorders such as PTSD.

INTRODUCTION

How the brain encodes high levels of fear is not fully understood. This question is

important because high levels of fear responding are associated with fear-related pathology (22;

47; 89; 90). Recent experimental data in human subjects suggest that individuals with post-

traumatic stress disorder (PTSD) have a higher 'fear load' compared to control subjects (89).

Experimental conditions inducing a conditioned fear memory in individuals with a higher fear

load leads to higher levels of fear response and reduced extinction of fear (89). The cellular and

molecular mechanisms underlying the acquisition and consolidation of an associative fear

memory are well-described (31; 45; 73; 83). In contrast, the cellular and molecular mechanisms

underlying divergent fear memory formation have seen less exploration. To date, the differences

in how individuals consolidate variable fear memory strength is unknown and this knowledge

may provide more targeted, effective treatments of fear memory disorders. In order to treat fear-

related neurobiological illnesses such as PTSD, it is important to understand the mechanisms

occurring in those individuals who exhibit strong fear learning or high 'fear load' (58; 89).

The formation of a long-term fear memory is generally considered to be an adaptive

neurobiological process that promotes survival (109). A process of learning involving well-

described and shared neural circuitry across a large number of species, the encoding and

consolidation of a fear memory requires key cellular signaling cascades in discrete brain regions

(71; 85; 113). These include the mitogen-activated protein kinase (MAPK) cascade in the lateral

amygdala (LA) (7; 119; 123; 129). In rodent models the phosphorylated (activated) form of

MAPK (pMAPK/ERK1/2) is required in the LA for the formation of long-term (but not short-

term) associative fear memory (29; 38; 118; 123). High and prolonged levels of fear response

characterize aspects of post-traumatic stress disorder (PTSD), and these increased responses may

be associated with aspects of underlying differences in the neurobiology of Pavlovian fear (58;

89).

In order to begin to identify some of the differences that underlie very low and very high

fear response, we used short-term selection to derive a mouse model selectively bred to show

robust differences in both contextual and cued Pavlovian fear memory (29; 94; 101). We refer to

these as "Low line" and "High line" mice. In order to determine the role of pMAPK in the High

and Low line mice, we pharmacologically inhibited the activation of pMAPK with the drug

SL327, a selective MEK inhibitor. In the High and Low line mice we tested whether contextual

and auditory cued fear memories are differently affected by the selective MEK inhibitor.

METHODS

High and Low Pavlovian fear mouse lines

Short-term selection for contextual fear was used to create outbred mouse lines with

robust differences in fear learning (101). Mice were phenotyped and selected at the Uniformed

Services University from a F_8 generation C57BL/6J x DBA/2J AIL (B6D2 F_8) obtained from the

University of Chicago (94; 101). Phenotyping was conducted by selecting the mice that

exhibited the highest and lowest contextual freezing one day following Pavlovian fear

conditioning (10-30% of populations selected to create new breeding generation). Freezing to the

CS was used as an additional measure for selection when necessary. Avoiding brother/sister and

first cousin pairings, high and low line breeding pairs were created and represented twelve

families per line. Offspring were fear conditioned at 8 weeks of age, selected for high and low

contextual fear and high and low line breeding pairs were again formed. This process continued

for 3 selection generations (S1-3). Behaviorally naïve, adult male mice from the 4th selected

generation (S4) were used for experiments described here. Mice were randomly assigned to one

of two major experimental cohorts which ran in parallel: SL327 or Vehicle (Veh).

Husbandry

Mice were housed 2-5 per cage segregated by sex and line (High or Low) in standard

shoebox cages in a climate controlled vivarium on a 12hr light/dark cycle with ad libitum food

and water. Behavioral experiments were conducted during the light cycle. All experiments

adhered to IACUC approved protocols and procedures were conducted in accordance with the

National Institutes of Health *Guide for the Care and Use of Experimental Animals.*

Pavlovian fear conditioning

Twenty-eight adult male mice (greater than 2 months, less than 20 months) were used for

all behavior experiments. Conditioning chambers (Coulbourn Instruments, Whitehall, PA)

measured 7"Wx7"Dx12"H and were inside sound attenuation chambers. Two distinct

environments were created with these chambers in order to represent box "A" and box "B".

Alterations to the environments involved changes in flooring (grid shock floor for training and

context test; wire mesh non-shock floor for cue test), chamber dimensions and appearance,

lighting, and scent (70% isopropyl alcohol cleaning solution versus commercial cleanser). Mice

were transported to a holding area free of high human traffic and noise and they remained in this

area for 30 minutes prior to being placed into the conditioning chambers. Behavior experiments

consisted of 3 days of habituation to "A", training day in "A", context test day in "A", and cue test day in "B" (6 consecutive days).

Pavlovian fear conditioning consisted of one paired tone/shock presentation (CS/tone: 75dB, 5000Hz x 30 seconds +US/foot shock: 0.5mA during final second of CS) over 5 minutes with 90-seconds following CS+US pairing prior to being removed from the box ("post-tone/shock"). Animals were allowed 3 minutes in the chamber prior to administration of any stimuli. One day following fear conditioning, animals were returned to the training context "A" for 5 minutes and freezing was scored. The animals were then returned to laboratory animal medicine (LAM) for one day. The following day, (two days following training), in order to isolate associative cued fear, mice were placed into a novel environment "B" and one tone (CS) identical in quality and duration to the training CS (75dB x 30 seconds) was administered. Three minutes elapsed in the novel environment prior to the administration of the CS and total testing time was 5-minutes.   Freezing, an active behavior, is the cessation of all movement except for movements associated with respiration (18). Freezing was measured using FreezeFrame™ (Coulbourn Instruments, Whitehall, PA) automatic scoring and verified by the investigator. Adjustments to threshold were made by the investigator who was blind to group assignment. These adjustments insured that automated software accurately detected freezing and threshold setting varied depending on mouse fur color (gray, light brown, or black) and lighting in each chamber.

Scoring of freezing

Freezing was measured across groups as follows: *Training Day*: During the 30-second tone/shock presentation and at 30-second intervals during the 90 seconds following the

tone/shock presentation; *Context Test Day*: at one minute intervals (T1-T5) for the 5 minutes of

testing, and *Cue Test Day*: during one minute intervals for the first 3 minutes of testing, during

the 30-seconds of the CS presentation, and during the 90-seconds following CS presentation

(post-tone). All data are means per group.

Pharmacology

SL327 (Sigma, St Lois, MO) was reconstituted in 100% dimethyl sulfoxide (DMSO) so

that dosing (100mg/kg) required approximately 40µl total volume. SL327 was received in

powder form and was reconstituted with 500µl 100% DMSO for a final concentration of

1mg/20µl. 100% DMSO in equivalent volumes was administered as vehicle. We found it

necessary to keep the total volume administered to less than 150µl (otherwise the mice became

sick presumably from DMSO). One hour prior to training, mice received an intraperitoneal

injection of either SL327 or DMSO and then returned to their home cage until training.

Immunohistochemistry

One hour following Cue Test, mice were anesthetized with ketamine/xylazine (100mg/kg

+ 10mg/kg) via intraperitoneal injection and transcardially perfused (Gravity Perfusion System

for mouse, AutoMate Scientific, Berkeley, CA) with 20ml 0.9% normal saline followed by 40ml

4% paraformaldehyde (FD Neurotechnologies, Columbia, MD). Brains were post-fixed

overnight and transferred to 1X phosphate buffered saline (PBS) until processing for

immunohistochemistry. Brains were sliced on a vibratome at 40µm and marked in a consistent

manner to indicate rostro-caudal sequence. The section selected for quantification of pMAPK

expressing neurons was bregma -1.70 as this is the section in which the LA subdivides into LAd,

LAvl, and LAvm (46). Only LAd was quantified as this discrete region was found to have

different quantities of pMAPK expressing neurons in the High and Low line mice (29). Free-

floating sections (5 per well) were placed in 1% BSA blocking solution for one hour prior to

incubation in rabbit polyclonal primary antibody to phospho-p44/42 MAPK (1:250 dilution, Cell

Signaling Technology, Boston, MA) x24 hours at room temperature. Following 5 washes in

PBS, sections were incubated in secondary antibody (biotinylated goat anti-rabbit IgG, 1:200

dilution, Vector Laboratories) for 30-minutes, washed x4, and then placed in avidin-biotin HRP

complex (ABC Elite, Vector Laboratories) for 1-hour. Staining of pMAPK-expressing neurons

was achieved by exposing sections to SG chromagen/hydrogen peroxide (Vector Laboratories).

Sections were mounted in sequence on Superfrost ™ slides, allowed to completely dry, and then

dehydrated in increasing percentages of alcohol (50% x1, 70%x1, 95%x1, 100%x2) followed by

xylene. Immunohistochemistry for pMAPK in rat and mouse models is an established technique

in our laboratory. Brain regions that serve as positive and negative controls (e.g. central nucleus

and optic tract respectively) are verified during each processing of tissue.

RESULTS

We find in a mouse line of high and low Pavlovian conditioned fear that inhibition of the

phosphorylation of MAPK with the MEK inhibitor SL327 reduces the strength of fear memory

to the conditioned context (context) and conditioned auditory cue (cue). We find that SL327 has

a more profound effect on context memory than on cue memory. Additionally, data suggest that

High line mice may be more susceptible to SL327 compared to Low line mice. High and Low

line mice were randomly divided into either drug (SL327; n=10 High line and n=4 Low line) or

vehicle (Veh; n=11 High line and n=3 Low line) group.

Acquisition of conditioned fear - training day

Training Day freezing revealed no differences in animals that received the drug (SL327) or vehicle (Veh) one-hour prior to training (see Figure 3.1). Freezing was measured for the 30.0 sec during tone presentation (which co-terminated with a 1.0 second foot shock) and during 3 x 30.0 sec post shock epochs (total 4 x 30.0sec epochs, 120 sec). A mean freezing score was calculated per group (percent freezing out of the total time measured).

High and Low line animals acquired different levels of Pavlovian fear during training. There was no significant effect of drug during training for either High or Low lines. A 2-way analysis of variance revealed a significant effect of Phenotype F $(1, 23) = 5.409$, p $= 0.0292$. Animals administered vehicle from the High line group showed 16.62 +/- 2.78 freezing compared to animals from the Low line group administered vehicle with 5.0 +/- 2.361. Post hoc analysis of High and Low line animals administered vehicle showed a significant difference on one-tailed t test p $= 0.0302$. These results suggest that the selected High line exhibits greater freezing during the acquisition of conditioned fear compared to Low line and that SL327 did not have a significant effect during training (Figure 3.1).

Context test

Upon return to the training context one day following training, mice that received SL327 one hour prior to training exhibited less freezing than mice that received vehicle (Figure 3.2). Freezing was measured for each minute during a 5 minute test. A mean freezing score was calculated (percent freezing out of 5 minutes). 2-way analysis of variance identified a significant effect of Drug F $(1, 24) = 9.013$, p $= 0.0062$. Post hoc analysis identified that High line animals were significantly reduced: SL327 0.33 +/_ 0.179 versus vehicle 23.12 +/- 5.19, one tailed t test p $= 0.0003$. Post hoc analysis identified that Low line animals were significantly reduced: SL327

0.7 +/- 0.702 versus vehicle 7.39 +/- 0.663, one tailed t test p = 0.0006. These data show that

SL327 significantly reduced freezing in both High and Low lines (Figure 3.2).

Cue test

In order to determine the degree to which the mice learned that the tone predicts a

footshock, they were individually placed into a novel conditioning chamber ("context B") and

administered one tone identical to that which was administered during training. Freezing was

measured for the 30.0 sec during tone presentation and during 3 x 30.0 sec post shock epochs

(total 4 x 30.0 epochs, 120 sec). A mean freezing score was calculated (percent freezing out of

120 seconds). 2-way analysis of variance identified a significant effect of Drug F (1, 24) =

4.494, p = 0.0445. Post hoc analysis identified that High line animals were significantly

reduced: SL327 9.25 +/- 3.27 versus vehicle 24.49 +/- 5.83, one tailed t test p = 0.0196. Post hoc

analysis identified that Low line animals were not significantly reduced: SL327 3.66 +/- 2.677

versus vehicle 15.509 +/- 6.59, one tailed t test p = NS (0.0609). These data show that SL327

significantly reduced freezing in High line but not Low line animals (Figure 3.3).

Normalized freezing to context versus cue

The data from context and cue memory tests (above) suggest that SL327 had more effect

on context memory than on cued fear memory for both the High and Low line animals. We

performed a secondary analysis in order to directly compare the effects of SL327 on contextual

and cued fear memory. As a measure of change resulting from SL327 we compared context fear

to cue fear following drug normalized to its own vehicle control. We identified a significant

difference between normalized High line SL327 context test 1.42 +/- 0.81 versus High line

SL327 cued test 37.79 +/- 14.1, two tailed t test p = 0.014274. In contrast there was no

significant difference between Low line SL327 contexts 23.6 +/- 19.94 versus Low line SL327

cued 10.15 +/- 10.96, two tailed t test p = NS (0.520185). These data indicate a potential

difference between High and Low line animals in their sensitivity to SL327, a systemically

administered MEK inhibitor (Figure 3.4).

pMAPK expressing neuron number in the LAd following cue test

In the final analysis we measured the effect of SL327 on the number of neurons

expressing pMAPK in one specific section of the dorsal sub nucleus of the lateral amygdala

(LAd) in High line animals following the recall of conditioned fear. Immunocytochemistry for

pMAPK identified pMAPK expressing neurons in both SL327 and Vehicle administered

animals. We found a significant difference in the number of pMAPK expressing neurons

between the SL327 animals: 2.500 ± 0.5000 n=2 versus the vehicle animals: 22.00 ± 1.732 n=3,

one tailed t test p = 0.0017. These data indicate that SL327 inhibited the activation of MAPK in

neurons in a specific section of the LAd (figure 3.5).

DISCUSSION

In these experiments we tested whether there was a different effect of pMAPK inhibition

(using the MEK inhibiter SL327) prior to Pavlovian fear conditioning between selectively bred

High and Low line mice (29; 94; 101). Second, we tested whether there was a different effect of

the pMAPK inhibition in different aspects of Pavlovian fear memory. We used freezing to

conditioned contextual and auditory cues as an index of memory acquisition, consolidation and

strength (29; 58). We found that the systemically administered MEK inhibitor SL327 did not

significantly affect the acquisition of Pavlovian fear during training. In contrast, long term

memory for contextual fear conditioning tested 24 hours following training was significantly

reduced in both High and Low line mice. For long term cued fear memory tested 48 hours

following training, High line mice but not Low line mice showed a significant reduction in fear

memory. We further explored this finding by comparing normalized levels of context and cue

fear memory following SL327 administration. We found that High line animals showed a

significantly different level of freezing between context and cue memory tests, while Low line

animals did not. Collectively these data suggest that while context memory is susceptible to

MEK inhibition in both High and Low lines, cued fear memory is susceptible only in High line

mice (29). We then asked whether these behavioral differences were associated with a reduced

number of pMAPK-expressing neurons in the LAd. We found a significant reduction in the

number of pMAPK expressing neurons in the LAd in SL327 treated animals compared to vehicle

controls. This finding suggests that the LAd, a key site for cued and contextual fear memory, is

directly affected by the systemic MEK inhibitor and that activation of MAPK is inhibited in

High line mice.

In the present experiments we confirmed the selected High and Low lines and further

identified key aspects of the cellular mechanisms that underlie the phenotypes (29; 58; 94; 101).

High line animals show more freezing during training compared to Low line animals and there is

no significant effect of drug. These data confirm the selected fear phenotype (29; 58; 94; 101)

and suggest that the MEK inhibitor is selective for the consolidation of memory (38; 119; 123).

We find that SL327 reduces contextual fear memory in both High and Low lines but cued fear

memory is significantly reduced only in High line mice. Importantly we find that SL327 can

reduce cue fear memory in High line animals into the range of fear memory of Low line animals

(Figure 3.3). These data suggest potential differences in sensitivity to inhibition between both

context and cued fear memory and importantly between High and Low lines. A technical

limitation of the conclusions may be the imbalance of High and Low line mice available to make

up the groups. On the other hand, the robust effect of SL327 on context memory in both

phenotypes compared to the reduced effect of SL327 on cued fear memory in the High line

animals and no significant effect of SL327 on cued fear in Low line animals may have relevant

implications. These findings suggest specificity for both a reduced effect of SL327 on cued fear

across the phenotypes and further suggest that High line animals may be more sensitive to a drug

that inhibits plasticity.

These data identify potential differences in sensitivity of cued fear memory compared to

context memory following MEK inhibition in a mouse model of High and Low Pavlovian

conditioned fear. Additionally, data suggest that higher fear individuals may be more sensitive

than lower fear individuals to the effect of MEK inhibition on cued fear memory. A potential

explanation for the difference in sensitivity to context versus cue memory may be that during the

breeding and selection of High and Low line mice, the primary selection of animals was based

on contextual freezing. Thus, we may have 'selected' lines with more robust genetic and cellular

differences driving contextual memory. Alternatively, it may be that the hippocampus is more

sensitive to the effects of SL327 as it is a much larger structure than the lateral amygdala. In

order to test the involvement of the lateral amygdala in the behavioral effects observed, we

measured the number of pMAPK expressing neurons in the dorso-lateral amygdala (LAd). These

anatomical data support the hypothesis that high fear is mediated, at least in part, by more

pMAPK in the LAd and that this increase is more sensitive to MEK inhibition. Collectively these

data suggest that higher fear individuals may be more sensitive to intervention or treatment with

MEK inhibitors compared to lower fear individuals, particularly in contextual fear memory.

Pavlovian fear conditioning has been proposed to underlie key aspects of memory

formation associated with anxiety disorders including PTSD (58; 138). Thus, understanding the

cellular mechanisms underlying Pavlovian fear conditioning has the potential to lead to the

development of targeted behavioral and pharmacological mechanisms aimed at inhibiting the consolidation and reconsolidation of fear memories (38; 39; 58). The MAPK cascade has been identified as an essential molecular pathway in Pavlovian fear consolidation in rats (12-14; 119), and mice (29; 38; 123) and also in reconsolidation of fear memories (14; 53). Recent experiments in humans with PTSD have identified increased strength of consolidated fear memory and a decreased rate of extinction (118). This increased Pavlovian fear memory strength, termed 'Fear Load' (118), suggests that individuals with PTSD may have different genetic and cellular mechanisms driving fear memory strength.

In these experiments we investigated an aspect of the 'fear load' hypothesis by testing whether animals known to develop different levels of Pavlovian fear memory are differently susceptible to inhibitors of MAPK memory consolidation cascade. The present data show that cued fear memory in High line animals is sensitive to inhibition by MEK inhibitors. This finding suggests that an increase in the number of pMAPK neurons or quantity of pMAPK is associated with the high fear phenotype. This conclusion is supported by our previous data showing that High line mice have a higher number of pMAPK expressing neurons in the LAd following Pavlovian fear conditioning (41). Collectively these data point to a hypothesis of an increased network of activated neurons in the LAd in High line mice and higher fear load individuals (56; 58). This increased network of neurons may suggest that an element of fear memory strength is governed by both the number and specificity of neurons encoding the fear memory in the LA (56; 58).

This study identified potential differences in distinct types of memory tests that may be more or less sensitive to pharmacologic inhibition. If a MEK inhibitor was used in a clinical setting for trauma associated memories, these data suggest that memories of the trauma context may be

inhibited more than memories of trauma-associated cues (i.e. sights, sounds, smells). More

studies in animal models are needed to further test and confirm these findings. These data

suggest that SL327 can be used in a mouse model of High and Low Pavlovian conditioned fear

to reduce high fear memory to the level of low fear memory. However, they also show that

contextual and cued fear memory may be differently susceptible to inhibition by MEK inhibitors.

Thus the usefulness of MEK inhibitors for clinical use may depend on the traumatic fear memory

being treated. Further work is needed to extend these finding and to explore the new hypothesis

raised by these data.
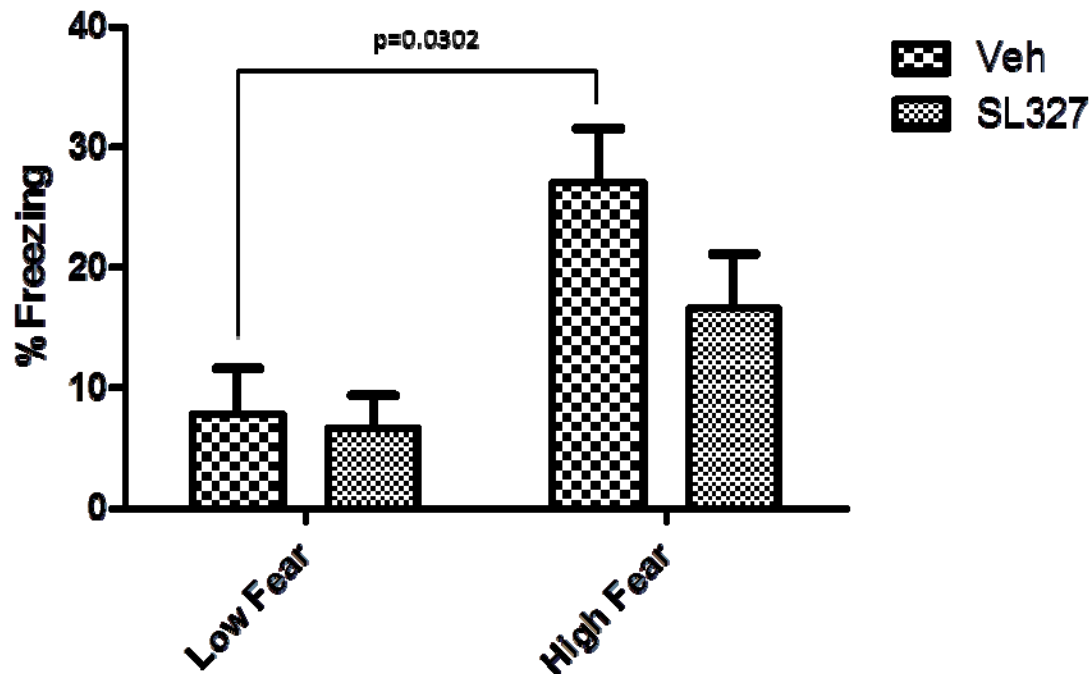
Figure 3.1. High and Low fear mice show different levels of fear memory immediately following
training. High fear vehicle control mice show more fear than Low fear vehicle control
mice. There was no significant effect of the MEK inhibitor SL327 during training. 2-
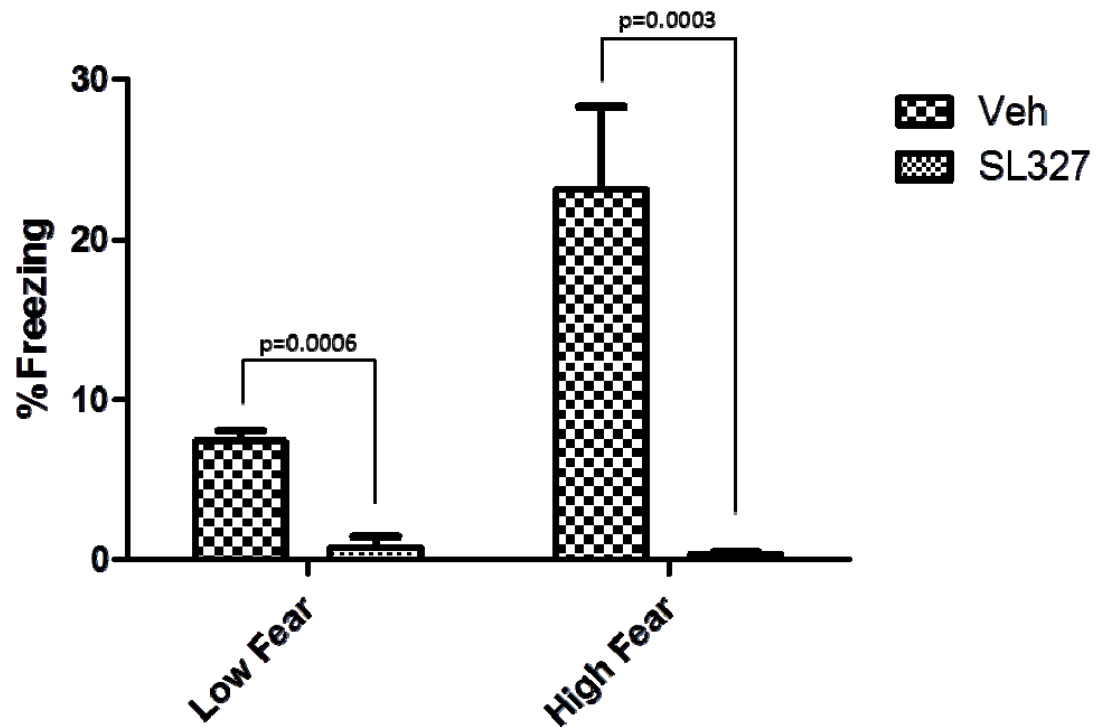way ANOVA reveals an effect of phenotype (p=0.0302).

Figure 3.2. The MEK inhibitor SL327 abolished contextual fear in both High Fear and Low Fear mice. SL327 was administered prior to initial fear conditioning. Contextual fear memory tested 24-hrs following initial fear conditioning consisting of 5 minutes in the training context. 2-way ANOVA reveals an effect of drug (p=0.0062).
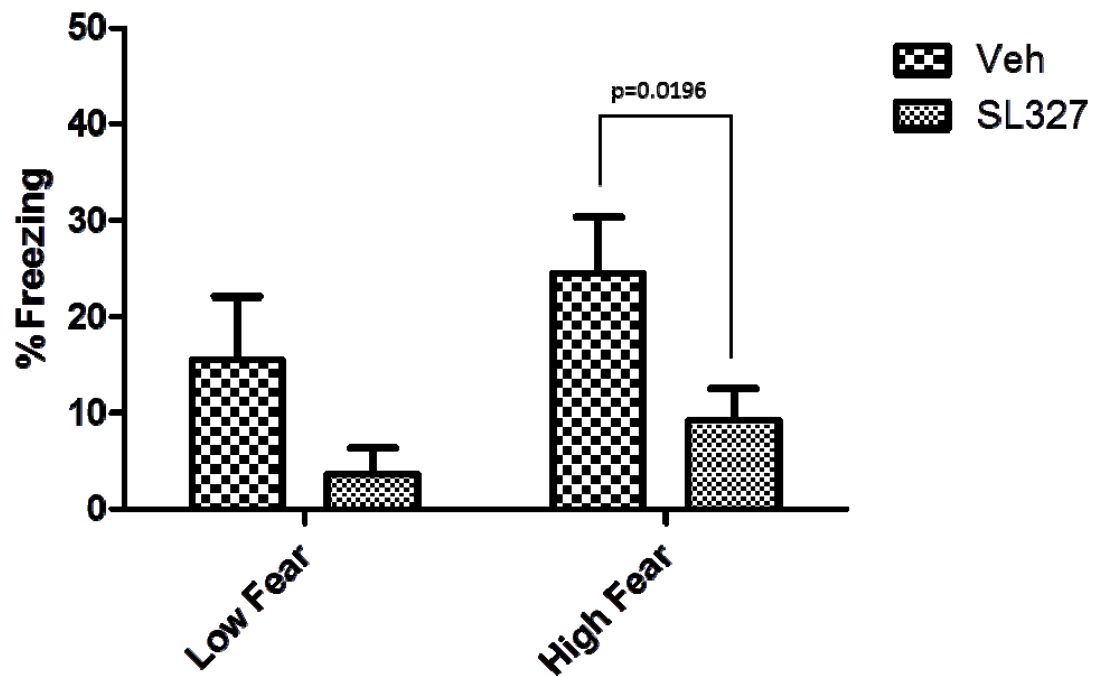
Figure 3.3. The MEK inhibitor SL327 inhibited cued fear conditioning in high fear mice. There was also a non-significant reduction in fear in Low Fear mice. Levels of fear to the conditioned cued were tested 48-hrs following initial fear conditioning. SL327 was administered prior to conditioning. 2-way ANOVA revealed an effect of drug (p=0.0445).
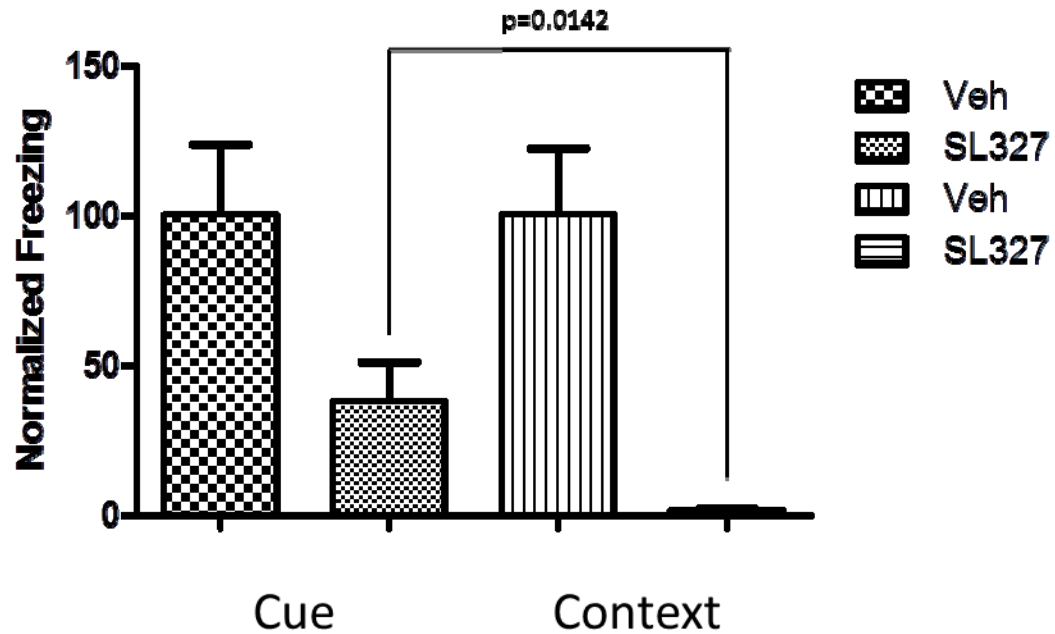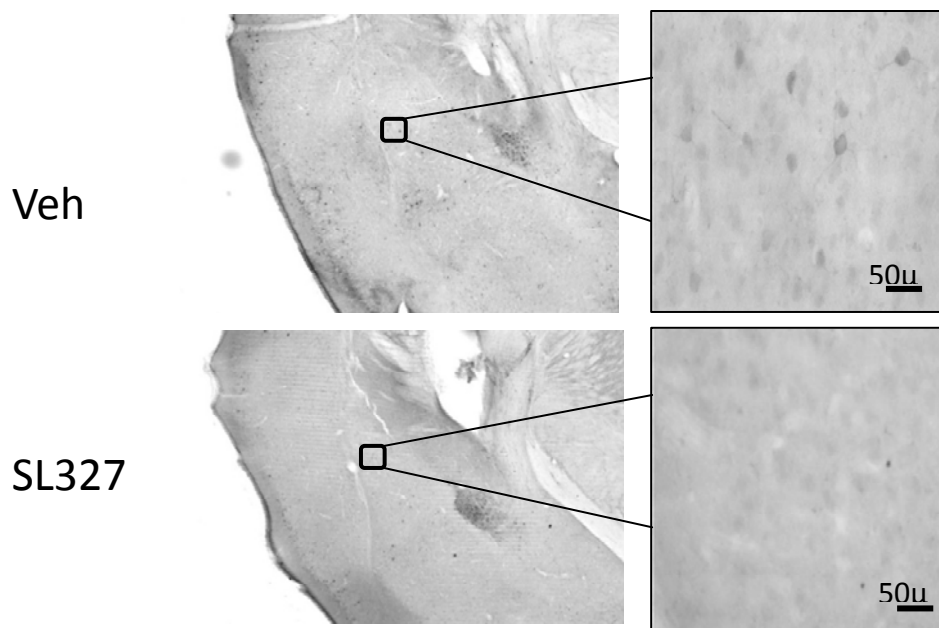
Figure 3.4. Comparison of the level of cued and context fear in High fear mice following SL327. Freezing is normalized to vehicle control. Post drug normalized Cued and Context fear measures are significantly different. Contextual fear is completely abolished by SL327 however cued fear was only reduced to ~ 50% of its control.
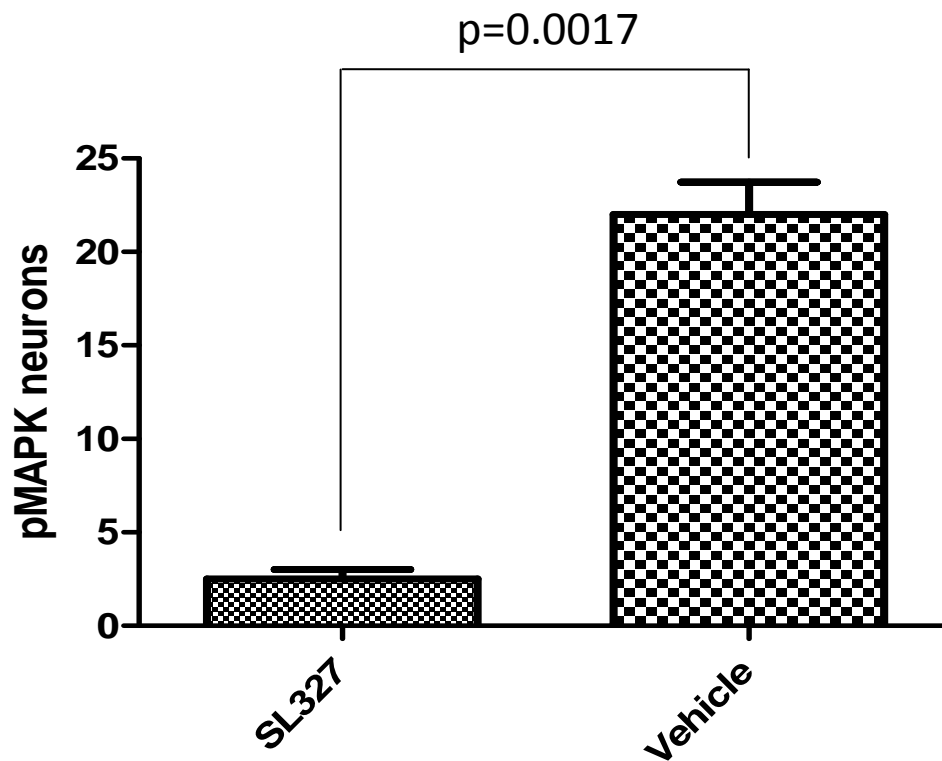
Figure 3.5. pMAPK-expressing neurons in the LAd one-hour following cue fear test
(reconsolidation) in mice that received SL327 (n=3) vs. mice that received vehicle
(n=3) prior to fear conditioning. The systemic MEK inhibitor SL327 reduces the
number of neurons expressing pMAPK in the lateral amygdala. In High fear animals,
60 min following cued memory test, the number of neurons in the dorsal sub-nucleus of
the lateral amygdala (LAd) expressing pMAPK, was significantly reduced.